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Beta-Globin Haplotypes From Blood Spots for Follow-Up of Newborn Hemoglobinopathy Screening

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The inheritance of sickle-cell anemia upon the background of the major β-globin gene cluster haplotypes has been associated with differing risks for major organ failure, and more recently with response to hydroxyurea treatment. Early identification of β-globin haplotypes in individuals with sickle-cell anemia may be a clinically useful prognostic factor for severity of disease expression. This report describes the use of whole-blood spots on filter papers from newborn hemoglobinopathy screening for β-globin gene cluster haplotyping by the polymerase chain reaction. Am. J. Hematol. 54:76–78, 1997.

Key words: β-globin gene haplotypes; hemoglobinopathy screening; sickle-cell anemia

INTRODUCTION

Early identification and treatment of children with sickle-cell anemia have been shown to decrease the morbidity and mortality associated with pneumococcal sepsis [1,2]. Consequently, the expansion of newborn screening programs to include hemoglobinopathy identification has been mandated in many states during the past decade. The California Newborn Screening Program began screening for clinically significant hemoglobinopathies in February 1990 [3,4]. Although the physiologic consequences of the genetic defect in the β-globin gene are highly variable, the majority of American sickle-cell anemia patients today progress beyond childhood. In older children and adults, therapeutic modalities such as transfusions, hydroxyurea treatment, and bone-marrow transplantations may be considered as the clinical condition warrants. Better radiologic imaging studies, including transcranial Doppler (TCD), magnetic resonance imaging and angiography (MRI and MRA), and positron emission tomography (PET), may help identify early the most adversely affected individuals, when therapeutic intervention can be most effective.

The inheritance of sickle-cell anemia upon the background of the major β-globin gene cluster haplotypes has been associated with differing risks for major organ failure, and more recently with response to hydroxyurea treatment. Patients with Central African Republic (CAR) haplotypes generally have a poorer clinical course [5] and are not highly represented in the group of sickle-cell anemia (SS) patients that respond well to hydroxyurea [6]. This study to determine the feasibility of identifying β-globin haplotypes on filter-paper blood spots was begun to provide easily obtainable, clinically relevant genetic information to referral area genetic center physicians.

MATERIALS AND METHODS

DNA Extraction

DNA was extracted from dried whole-blood specimens as previously described [7]. One half of a 1.0–1.2-cm dried blood spot on filter paper (Schleicher & Schuell,

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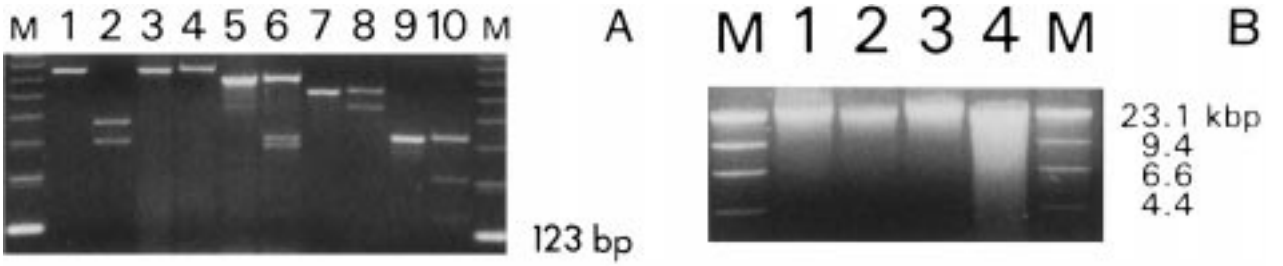

Fig. 1. Beta-globin haplotype determinations from blood spots using PCR. A: Three percent agarose gel separation of PCR reactions at beta-globin cluster polymorphic sites as follows: $^{G}\gamma$, $^{A}\gamma$, $\psi\beta$, $3'\ \psi\beta$, and $5'\ \beta$, undigested (lanes 1, 3, 5, 7, and 9, respectively); *Hind*III-digested $^{G}\gamma$ and $^{A}\gamma$ (lanes 2 and 4, homozygous +/+ and −/−, respectively); *Hinc*II-digested $\psi\beta$ and $3'\ \psi\beta$ (lanes 6 and 8, heterozygous −/+ for each); and *Hinf*I-digested $5'\ \beta$ (lane 10, heterozygous −/+). Lane M, 123-bp ladder. Patient haplotype is heterozygous CAR/Sen (+−−−−/+−+++). B: Lanes 1–4 represent 10% of the extracted DNA from one half of a 1.0-cm diameter, $5\frac{1}{2}$-year-old dried blood spot on filter paper from four SS patients. Lane M, *Hind*III-digested lambda DNA size marker (200 ng). Although specimen 4 shows some degradation, PCR reaction results were not affected.

Keene, NH or 3MM Whatman, Maidstone, England) from either 50 μl of EDTA-anticoagulated whole blood or a newborn screening specimen was cut into small pieces and placed into a 1.5-ml sterile microcentrifuge tube. After fixation of hemoglobin to the filter paper with several drops of methanol for 5 min, the sample was dried under vacuum. Four hundred and eighty μl of 0.15 M NaCl, 0.5% SDS, were added, and incubated at 37°C for 1 hr, followed by addition of 40 μl proteinase K (20 mg/ml), and incubation overnight at 37°C. The mixture was subjected to three phenol extractions (500 μl × 1, 250 μl × 2) and two chloroform/isoamyl alcohol (24:1) extractions (250 μl). DNA was precipitated with 1/10 volume 3 M potassium acetate and 1 ml of 100% ethanol (−20°C, 1 hr to overnight) prior to centrifugation (16,000*g*) for 20 min. Pelleted DNA was washed once with 70% ethanol, air-dried, and resuspended in 100 μl 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Ten μl were used as template for polymerase chain reaction (PCR) amplifications. Alternatively, methanol-fixed blood spots were resuspended in 61 μl dH_2O, boiled for 15 min, and centrifuged at 16,000*g* for 20 min. Ten μl were used as template for PCR amplifications.

PCR Reactions

Polymerase chain reactions for the $^{G}\gamma$, $^{A}\gamma$, $\psi\beta$, $3'\ \psi\beta$, and $5'\ \beta$ polymorphic sites in the beta-globin gene cluster were done following a previous method [8], with minor changes to cycling parameters to optimize specific products. The determination of these five polymorphic sites by PCR have been shown in this laboratory to correspond to previous determinations by Southern blot (data not shown). In comparison to previous studies [9], the use of the *Hinf*I site instead of the *Hpa*I site near the beta-globin gene to assign haplotypes corresponds well and better differentiates the hemoglobin C-related and Saudi haplotypes, and obviates the problems identified with amplifying repetitive sequences around the *Hpa*I site [10]. Restriction enzyme digests were done with *Hind*III, *Hinc*II, and *Hinf*I (New England Biolabs, Inc., Beverly, MA) overnight with an excess of enzyme at 37°C. Agarose gel (1–3%) electrophoresis in 1 × Tris-acetate/EDTA buffer was at 10 V/cm for 30–60 min.

RESULTS

Initially, 50 μl of whole-blood samples from 10 consecutive, previously haplotyped patients visiting the University of Southern California Comprehensive Sickle Cell Center was spotted onto filter paper, dried, and then extracted for DNA. Using primers that flank five commonly used restriction sites in the β-globin cluster, DNAs were amplified by polymerase chain reaction (PCR) and then digested with the appropriate restriction enzyme overnight. Agarose gel analysis identified homozygosity (−/− or +/+) or heterozygosity (−/+) for the presence of polymorphic sites. Figure 1A shows the analysis of one patient's heterozygous CAR/Sen haplotype as an example. All 10 samples showed identical haplotypes to those previously determined. Both described DNA extraction methodologies were successfully used, although DNA template derived from the more rapid boiling procedure was less reliably successful and occasionally required repeat analysis.

The DNA from four $5\frac{1}{2}$-year-old blood spots submitted to the California Newborn Hemoglobinopathy Screening Program was then extracted and run on a 1.0% agarose gel (Fig. 1B). This analysis showed remarkably good yield and predominantly intact high molecular weight DNA, although partial degradation in one sample could be seen. PCR reactions were done as above, with analogous results demonstrating that the PCR amplification product was not affected by the degree of partial degradation seen in Figure 1B. All four samples were haplotyped without difficulty, resulting in the unambiguous assignment of

two Ben/Ben and two Sen/Ben combinations (data not shown).

DISCUSSION

Polymerase chain reaction (PCR) identification of haplotypes performed on dried blood spots presents a rapid, simple method to provide β-globin haplotype data on newly diagnosed SS patients for clinical follow-up and study in conjunction with newborn screening programs. For other study populations, these methods combine well to obviate the problems encountered with the drawing, packaging, and shipping of blood samples from one location to another for haplotype determinations. A couple of drops of whole blood from a routine CBC tube or a fingerstick dried on a filter paper can be sent covered in plastic conveniently through the mail and usefully stored at room temperature for years.

Haplotype data are becoming clinically relevant to the selection of treatment with hydroxyurea or bone-marrow transplantation, as well as the timing of radiologic CNS studies or neuropsychiatric testing. In addition, counseling regarding prognosis and the expected clinical course can be provided early in life and potentially improve the early diagnosis of major complications (e.g., stroke) and allow for useful long-term family and medical management planning.

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REFERENCES

1. Gaston MH, Verter JI, Woods G, Pegelow C, Kelleher J, Presbury G, Zarkowsky H, Vichinsky E, Iyer R, Lobel JS, Diamond S, Holbrook CT, Gill FM, Ritchey K, Falletta JM: Prophylaxis with oral penicillin in children with sickle cell anemia: A randomized trial. N Engl J Med 314:1593, 1986.
2. Vichinsky E, Hurst D, Earles A, Kleman K, Lubin B: Newborn screening for sickle cell disease: Effect on mortality. Pediatrics 81:749, 1988.
3. Shafer FE, Lorey F, Cunningham GC, Klumpp C, Vichinsky E, Lubin B: Newborn screening for sickle cell disease: 4 years of experience from California's newborn screening program. J Pediatr Hematol Oncol 18:36, 1996.
4. Lorey FW, Cunningham GC, Shafer FE: Universal screening for hemoglobinopathies using high-performance liquid chromatography: Clinical results of 2.2 million screens. Eur J Hum Genet 2:262, 1994.
5. Powars D, Hiti A: Sickle cell anemia: β^s gene cluster haplotypes as genetic markers for severe disease expression. Am J Dis Child 147:1197, 1993.
6. Steinberg MH, Lu Z-H, Barton F, Terrin M, Charache S, Dover G: Fetal hemoglobin (Hb F) in sickle cell anemia (HbSS): Early and sustained effects of hydroxyurea. National Sickle Cell Disease Annual Meeting Program. Abstract O13, 1996.
7. McCabe ERB: Utility of PCR for DNA analysis from dried blood spots on filter paper blotters. PCR Methods Appl 1:99, 1991.
8. Sutton M, Bouhassira EE, Nagel RL: Polymerase chain reaction amplification applied to the determination of β-like globin gene cluster haplotypes. Am J Hematol 32:66, 1989.
9. Schroeder WA, Powars DR, Kay LM, Chan LS, Huynh V, Shelton JB, Shelton JR: β-cluster haplotypes, α-gene status, and hematological data from SS, SC, and S-β-thalassemia patients in Southern California. Hemoglobin 13:325, 1989.
10. Wang X, Bouhassira EE: *Hpa*I polymorphic site 3′ of the human β-globin gene is inside a repetitive sequence and cannot be ascertained by polymerase chain reaction. Am J Hematol 39:226, 1992.